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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

Docket Number	26250	Type a plus sign (+) inside this box ->	+

INVENTOR(s) / APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
SAUOB	Sobhi		Shibli Village - Lower Galilee, Israel
van MIL	Jan		Givat Nili - D.N. Menashe, Israel

TITLE OF THE INVENTION (280 characters max)

HERBAL EXTRACT FOR THE REDUCTION OF GLUCOSE LEVELS IN BLOOD

CORRESPONDENCE ADDRESS

G. E. EHRLICH (1995) LTD.
c/o ANTHONY CASTORINA
2001 JEFFERSON DAVIS HIGHWAY
SUITE 207

22581 U.S. PTO
60/530918
122203

STATE	VIRGINIA	ZIP CODE	22202	COUNTRY	USA

ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification	*Number of Pages*	11	☑ Applicant is entitled to Small Entity Status
☑ Drawing(s)	*Number of Sheets*	8	☑ Other (specify) *ASSIGNMENT* 7 CLAIMS

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☐ A check or money order is enclosed to cover the filing fees		FILING FEE AMOUNT ($)	$ 80.-
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

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Respectfully submitted,

SIGNATURE ____________________

December 18, 2003
Date

25,457
REGISTRATION NO.
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TYPED or PRINTED NAME **SOL SHEINBEIN**

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HERBAL EXTRACT FOR THE REDUCTION OF GLUCOSE LEVELS IN BLOOD

Inventors: Sobhi Sauob and Jan van Mil

Indigenous Herb for the Reduction of Glucose Levels in Blood

1. Introduction

i. Diabetes

Diabetes is a general term for a group of diseases which are characterized by the improper interaction of the body with the hormone insulin: either it is not available at all, or not in sufficient quantities, or it is not utilized.
There are two main forms of diabetes, Type I: juvenile, or Insulin-Dependent-diabetes, and Type II: Adult-onset or Insulin-Independent-Diabetes. The former is an autoimmune disease which results in the rapid destruction of beta-cells in the pancreas, which leaves he patient without the ability to produce insulin. The latter disease is characterized by different developments, which range from the inability to produce sufficient insulin to difficulties in utilizing insulin, expressed in problems with the transport of the hormone from the bloodstream through the cell membrane.

All types of diabetes are characterized by the inability to control the level of glucose in the bloodstream. Both hyperglycemia (high glucose blood levels) as well as hypoglycemia (low blood glucose levels) is dangerous conditions that have both immediate and long-term health consequences. Thus, a major issue in diabetes is the frequent measurement of glucose levels and their adjustment when necessary. Adjustment is done through the injection of insulin in Type I diabetes, while Type II patients have a range of products available to them which effect different stages of the complicated process of glucose consumption.

ii. Herbal Medicine

Medicine based on herbs and herbal products has found extensive application all over the world, from China to India, from the American India population to Middle Eastern population groups. Many of its products have found their way to the Western World and quite a few of them formed the basis for potent and successful pharmaceutical products.

The Arab population of Israel has through the years made extensive use of herbal medicine products and to this day many people use herbal products, dispensed by indigenous practitioners for a wide variety of ailments.

The potential of such Herbal medicine products to become "conventional" pharmaceutical products, or even only food supplements (nutraceuticals), depends on standard testing that must be performed on such product, to prove both efficacy and safety.

iii. Diabetes and Herbal Medicine

A large number of products is available as purported treatment for Diabetes (mostly Type II), that have their origins in the traditional medicine from various parts of the globe. Even though many of these are known and have been used for generations, their efficacy has not been scientifically proven and in some cases even, evidence exists that the materials have no effect whatsoever.

This leads to problematic developments with patients, and for instance, the American Diabetic Association has issued standards for research of therapies, and advises the diabetic public to judge products according to these standards, which include proper clinical research and peer reviewed publications of results. Also the Israeli Diabetes Association, through its chairman, Prof. Itamar Raz, uses as its motto "Science-Based Medicine".

Thus it is of major importance to be able to show the safety and efficacy of a natural product, also when it is known in the herbal medicine world for generations.

2. The Plant Portulaca Oleracea L.[1]

Portulaca Oleracea L., (Plant B) is a very common weed (Common names: Purslane, Verdolaga, Pursley) and found in most part of the world.

Plant B is considered a GRAS weed and in various parts of the world, including the Galilee area of Israel, it is grown and eaten as addition to green salads.

Plant B contains many biologically active compounds as well as many nutrients, including alkaloids, omega-3 fatty acids, coumarins, flavonoids and anthraquinone glycosides[2].

The plant has been used traditionally for a wide variety of ailments, in particular as a treatment for parasites, and for digestive disorders. In addition, anti-inflammatory and anti-fungal activity has been reported. Untested reports from around the world attest to the use of purslane as a remedy for many ailments and conditions[3].

Plant B has been mentioned in various sources as a remedy for hyperglycemia, but only one reference mentions experiments performed to show its efficacy in the reduction of glucose levels in blood.[4]

While reducing the present invention to practice the present inventors uncovered that fractions, particularly an alcohol and water extract, of Portulaca Oleracea L., (Plant B) can be efficiently used to reduce glucose blood levels in individuals in need thereof in a biosafe manner.

4. Results

i. Preliminary Results obtained on humans

This part of the study was conducted in cooperation with physicians and biochemical laboratories in the Nazareth region. Two groups were tested for the evaluation of the effect of Plant B on blood glucose levels. The blood glucose level in the patients of the first group was higher than 300mg/dl at the start of the trial, while the level of blood glucose in the second group was less than 300mg/dl. Plant B was administered at a level of 450 mg/day without any other medication. In both groups a remarkable reduction in blood glucose level was observed. In addition the patients reported a positive feedback. These results showed that those diabetic patients with less than 300mg/dl-blood glucose maintained normal blood glucose levels after 2-3 weeks. In the group of diabetic patients with more than 300mg/dl blood glucose normal blood glucose levels were attained after 4-5 weeks.

Table 1&2 show the characteristics of the patients involved in the trial and figures 1&2 show the results.

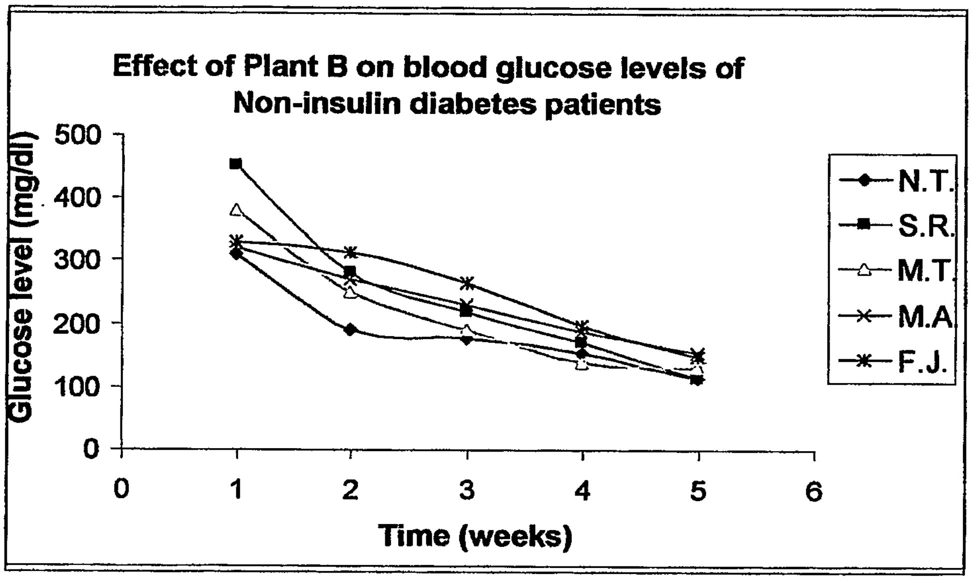
Table 1: The clinical history of the patients, with more than 300 mg/dl blood glucose.

No	The name	Weight kg	Age	Sex	Presence of other diseases	Diabetes History	Hb1C at beginning
1	N.T.	83	57	F	-	16	-
2	S.R.	95	64	M	High blood pressure	11	-
3	M.T.	89	62	M	-	7	-
4	M.A.	92	59	M	Cholesterol	10	-
5	F.J.	75	55	F	-	3	-

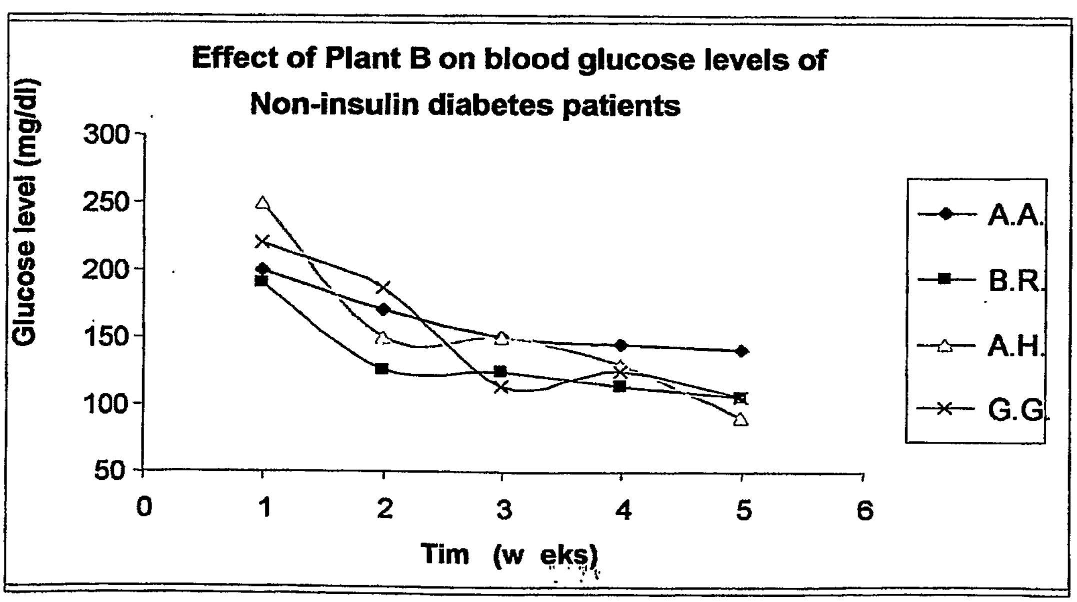
Table 2: The clinical history of the patients, with <u>less</u> than 300 mg/dl blood glucose.

No.	The name	Weight kg	Age	S x	Pre nce of other diseases	Diab tes History	Hb1C at beginning
1	A.A	82	47	M	-	5	-
2	B.R.	85	67	F	-	8	-
3	A.H.	107	58	F	High blood pressure	3	-
4	G.G.	96	72	M	Cholesterol	14	-

<u>Figure 1: Diabetic patients with more than 300mg/dl blood glucose</u>



<u>Figure 2: Diabetic patients with less than 300mg/dl blood glucose</u>



The two graphs summarize the activity of Plant B on two groups of diabetic patients.

The choice of the two groups was done on the basis of the level of glucose in the blood. The results show that the product is capable of normalizing the glucose blood level in both groups.

ii. In Vitro Toxicology Studies[5]

The biosafety of Plant B was evaluated using in vitro cell culture techniques. Therefore, the effects of the test plant extracts were investigated in human hepatocyte cell line (HepG2) and in co-cultures of hepatocytes and monocyte cell line (THP1). In addition, lipopolysaccharides (LPS), a known activator of macrophages and hepatocytes, stimulated cells were treated with various concentrations of plant extracts. The following end points were measured: Cell viability and cytotoxicity (MTT test and LDH release) and the expression of differentiated function in hepatocytes (albumin secretion). In addition, an MDA test was performed. This assay is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The effects of the test plant extracts was investigated in sheep liver tissue homogenate. All experiments were carried out in triplicates and each test was repeated three times.

Results obtained here indicate that extract of plant B was found to be bio-safe at concentrations up to 1000 g/ml in all end points measured including MTT test, LDH release, and albumin secretion. The MDA tests indicate that plant B has no effect on free radicals, and shows no effect at all in reducing the free radical concentration, but also does not enhance their occurrence.

Taken collectively, no sign of any toxic effects was found with extract from plant B in all parameters measured up to concentrations of 1000 g/ml.

MATERIALS

Cell culture: The potential toxicity of the plant extract was assessed in a cell culture system using the human hepatoplastoma cell line HepG2 cells and monocyte cell line THP1. HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes and can be grown indefinitely, thus permitting long-term studies to be performed. The cells from both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose content (4.5 g/L) supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10μg/mL streptomycin.

Cells were maintained in humidified atmosphere of 95% O_2-5% CO_2 at 37°C. The pH of the media was monitored at 7.4. The medium of cells from both cell lines was changed twice a

week. At 70-80% confluence, cells were trypsinized and plated in microtiter dishes. 24h after cell seeding, cells were exposed to various concentrations of the plant extracts in fresh serum-free medium.

Methods

1. *MTT assay*

The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of the cells. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the red formazan derivative by mitochondrial succinate dehydrogenase in viable cells. $2x10^4$ cells/100µL in each well of 96 well plates were seeded. Twenty four hours after cell seeding, cells were incubated with varying concentrations of water extracts of the four plants for 24 hours at 37°C. Following the removal of the plant extracts from each well, cells were washed in phosphate buffered saline. The cells were then incubated in serum free DMEM to which MTT (0.5mg/mL) was added to each well (100µL), and incubated for a further four hours. Then the medium was removed and the cells are incubated for 15 minutes with 100µL of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the MTT formazan is determined at 570 nm in an Elisa reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

2. *Lactate dehydrogenase*

In the Lactate dehydrogenase (LDH) assay the leakage of the cytoplasm located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium is indicative of cell membrane damage.

For the LDH assay, $2x10^4$ cells in 100µL of medium are seeded in each well of 96-microtiter plate. Twenty four hour after cell seeding, cells were exposed to varying concentrations of the plant extracts (0.001-0.5mg/mL). After 24h of treatment, the supernatants were collected from each well. Cell monolayers were then treated with a cell lysis solution for 30 minutes at room temperature to lyse. The cells and the lysate were collected. LDH activity was measured in both the supernatants and the cell lysate fractions by using CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega, WI, USA) in accordance with the manufacturer's instruction. The general chemical reactions of the assay are as follows:

$$NAD^+ + \text{lactate} \xrightarrow{LDH} \text{pyruvate} + NADH$$

$$NADH + INT \longrightarrow NAD^+. + \text{formazan (red)}$$

The assay to measure LDH activity is based on the conversion of tetrazolium salt into a red formazan product. The intensity of the color is proportional to LDH activity. The absorbance is

determined at 490 nm with 96-well plate ELISA reader. The percent of LDH release from the cells was determined using the formula:

LDH release = (Absorbance of the supernatant)/(absorbance of the supernatant + lysate)*100.

3. LPS treatment

For the evaluation of the effects of plant extract on LPS activated cells, hepatocytes and co-cultures of hepatocytes ($2x10^4$) with monocytes ($5x10^3$) were seeded in 100□l in each well of 96 well plates. Twenty four hours after cell seeding, cells were incubated with varying concentrations of the four plants in the absence and the presence of 10□g LPS/□l for 24 hours at 37°C. MTT and LDH release were carried then out as described above.

4. Quantification of albumin secretion

The amount of albumin in the culture supernatant was measured using enzyme-linked immunosorbent assay (ELISA). In brief, supernatants were incubated in 96-well microtiter plates for 1 hour at 37°C or overnight at 4°C. After washing in PBS, non-specific binding sites were blocked in PBS containing 0.5% bovine serum albumin (BSA) for 1 hour at room temperature.

After another washing step in PBS, peroxidase-conjugated goat anti rat albumin antibody was added in PBS containing 1% BSA and incubated for 2 hours at room temperature. The microtiter plates were then washed, the substrate (0.5 mg 2.2-azino-di-3-ethylbenzothiazoline-6-sulfonic acid per ml 100 mM Na-acetate, 50 mM Na-phosphate and 9 x 10^{-3} % H_2O_2) added and the absorption was measured at 405 nm in an ELISA reader. All washing steps were carried out with PBS at room temperature.

Background values were measured in the absence of culture supernatant and subtracted from the experimental values. All ELISA determinations were carried out in duplicates.

5. MDA Assay[6]

The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (1-3). This rapid, easy-to-use procedure has been modified by researchers for use with many types of samples including drugs, food products and human and animal biological tissues (4-7). The assay has provided important information regarding free radical activity in disease states and has been used for measurement of anti-oxidant activity of several compounds

A TBARS solution was prepared as follows: 0.12 M TBA, pH=7.0 (0.375 gr TBA, 2.5 ml HCl conc., 15 ml 100% TCA (TriChloroAceticAcid) , fill with water to 100 ml). Heat the solution (40

▯ ▯ °C approx.) till the TBA dissolves. The MDA standard solution: 82 µl MDA add 3.5 ml HCl conc. Make up to 50 ml with saline (0.9 % NaCl) we get 10 mM MDA solution. The working solution is a 100-fold dilution of this standard.

The samples are analyzed via O.D. at 522 nm versus water blank.

Results

1. Evaluation of the biosafety of the test extracts using MTT test:

A. HepG2 cells

The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of the cells. To evaluate the biosafety of the four extracts HepG2 cells were incubated with varying concentrations of the plant extract for 24h. Following the removal of the plant extracts from each well, cells were washed in phosphate buffered saline, and the MTT assay was carried out as described. Extract from plant B exhibited no sign of any negative effects at all concentrations tested.

B. Cocultures of HepG2 with THP1

To evaluate the biosafety of the four extracts HepG2 cells were cocultured with monocyte cell line THP1 and incubated with varying concentrations of the four plants extracts for 24h. Following the removal of the plant extracts from each well, cells were washed in phosphate buffered saline, and the MTT assay was carried out as described. Extracts from plant B exhibited no sign of any negative effects at all concentrations tested.

C. Cocultures of HepG2 with THP1 in the presence of lipopolysacchride (LPS)

To evaluate the biosafety of the four extracts HepG2 cells were co-cultured with monocyte cell line THP1 and incubated with varying concentrations of the four plants extracts for 24h in the absence and presence of LPS.

2. Evaluation of the biosafety of the test extracts using LDH-release test:

A. HepG2 cells

Membrane integrity can be evaluated by measuring the lactate dehydrogenase activity. Lactate dehydrogenase, an enzyme located in the cytoplasm, catalyses the conversion of lactate and pyruvate. When lactate dehydrogenase is found within the media on the cells, there are two possible causes: The first is cellular death and the second may be a 'leak' in a cell membrane. When cells are disrupted, the lactate dehydrogenase activity is elevated.

Result obtained in the present in vitro study indicate that treatment of HepG2 cells with plant extract B for 24h induce no significant change of the LDH levels in the culture medium.

B. Cocultures of HepG2 with THP1: As seen with HepG2, LDH levels were not significantly increased after treatment with extract B.

C. Cocultures of HepG2 with THP1 in the presence of lipopolysacchride (LPS)

As seen in the MTT test, extract B was found not affect the cell viability.

3. Evaluation of the bio-functionality of HepG2

The effect of the plant extract on the expression of liver specific function was measured by assessing the albumin secretion by HepG2. The levels of albumin in the cell culture medium was found to be unchanged by the treatment with the plant B test extract.

4. MDA test

The MDA release was measured as a function of plant extract concentration and no effect was registered, indicating that the extract has no effect, not positive and not negative, on the peroxidation processes.

<u>Figure 3: MTT Assay in HepG2 cells after an overnight incubation with various concentrations of test plant extracts</u>

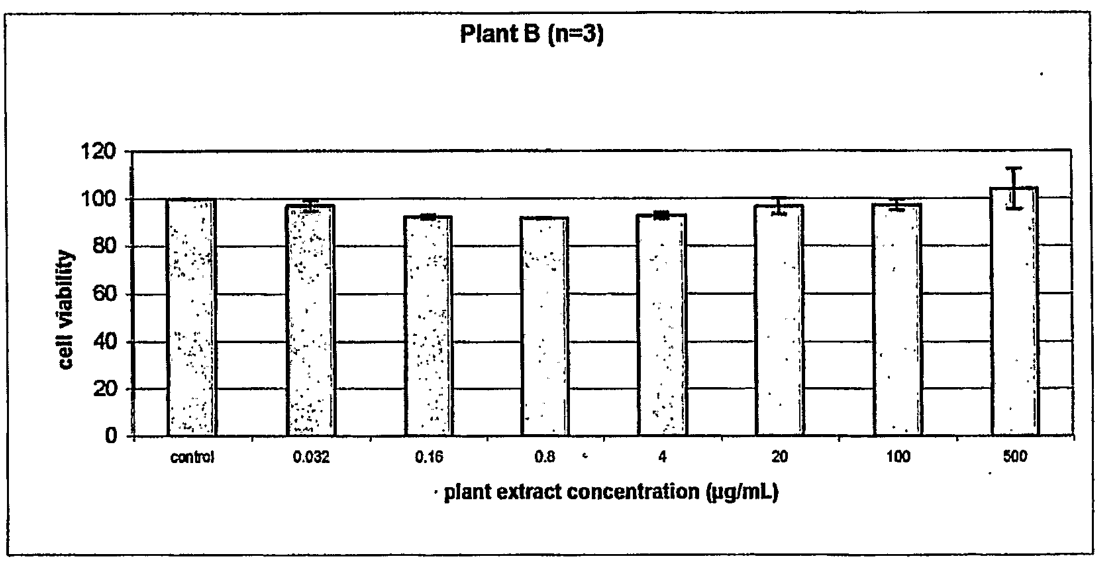


Figure 4: MTT assay in th co-culture (HepG2 & THP1 c lls) after overnight incubation with various concentrati ns of th test plant extracts.

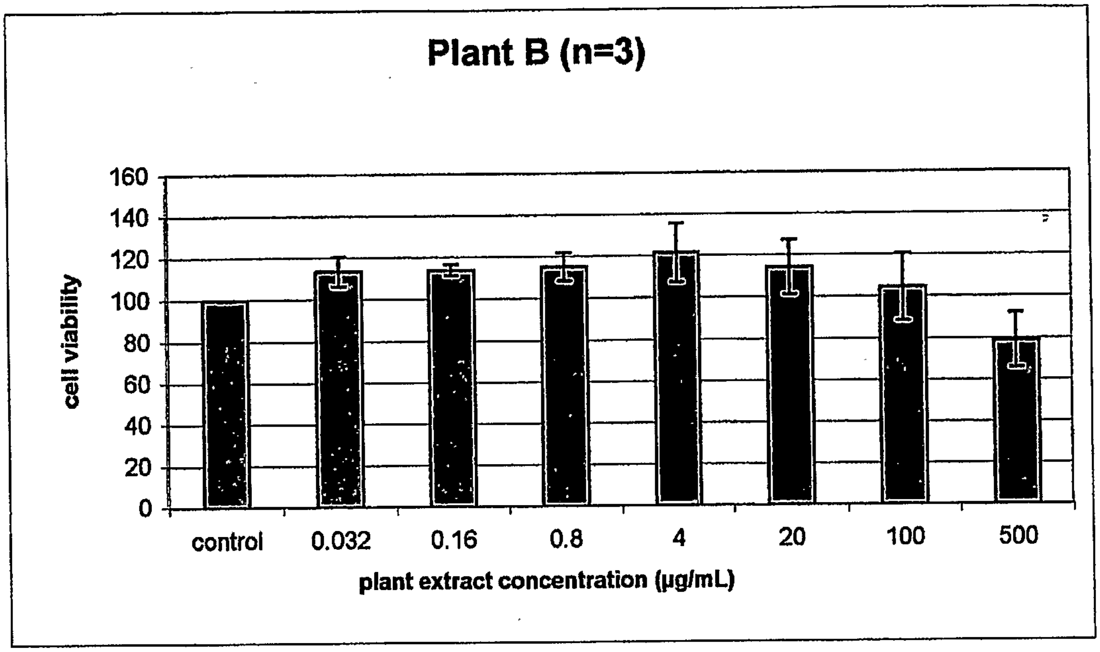


Figure 5: LDH leakage as % of total from HepG2 cells after an overnight incubation with various concentrations of the test plant extracts.

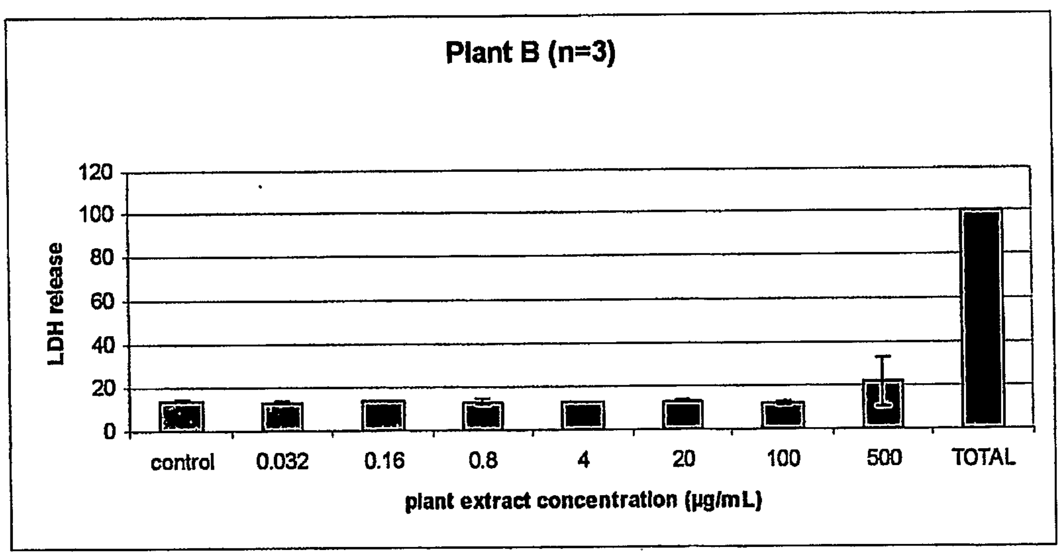


Figure 6: % LDH leakage from HepG2 cells after an overnight incubation with various concentrations of the test plant extracts.

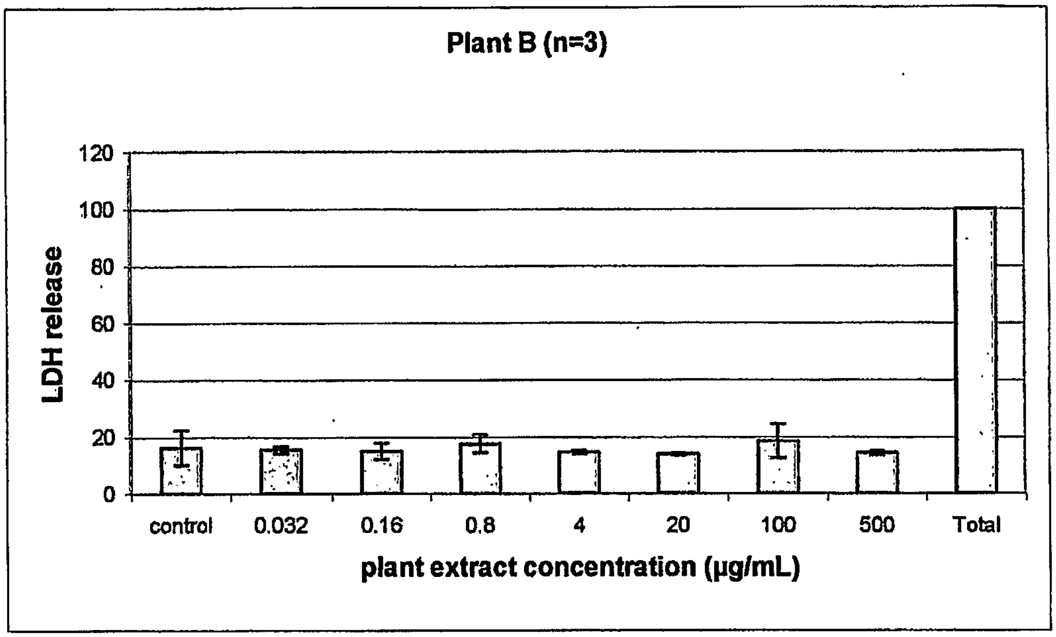


Figure 7: Albumin production in HepG2 cells after an over night incubation with various concentrations of the test plant extracts plant

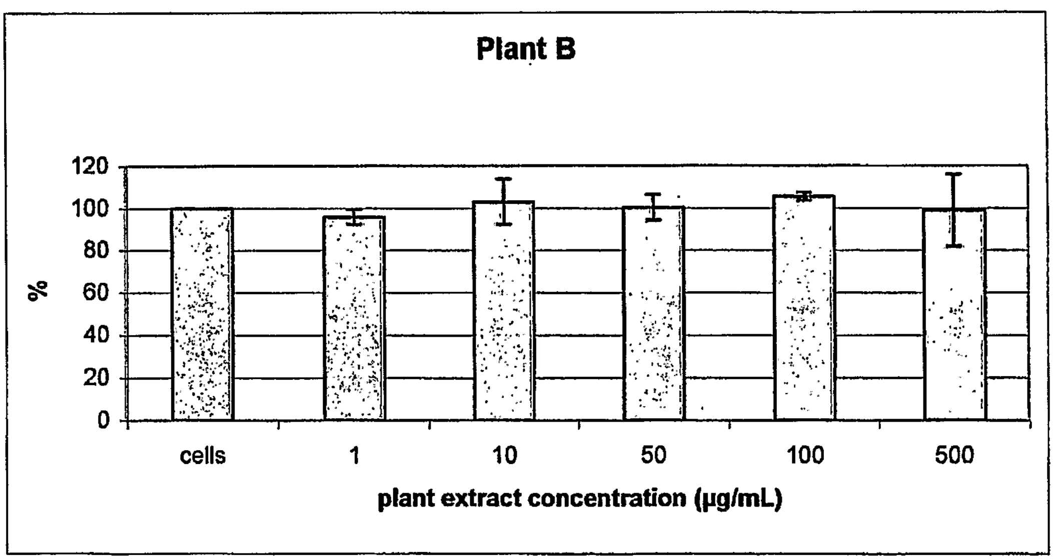


Figure 8: MTT assay in the (HepG2 & THP1 c lls) after an overnight co-incubation with 10 µg/mL LPS and various concentrations of the test plant extracts

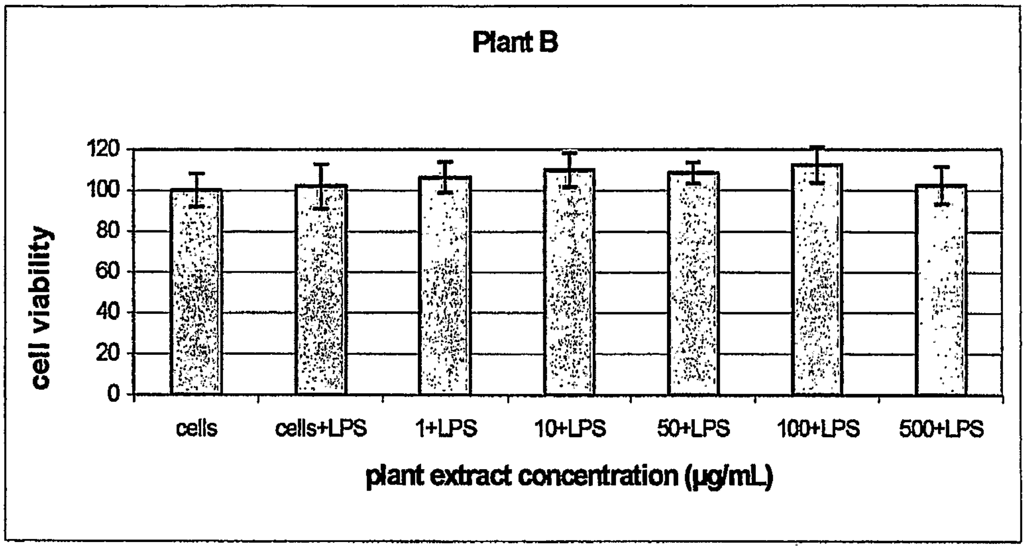


Figure 9: % LDH leakage from HepG2 & THP1 cells after an overnight co-incubation with 10 µg/mL LPS and various concentrations of the test plant extracts

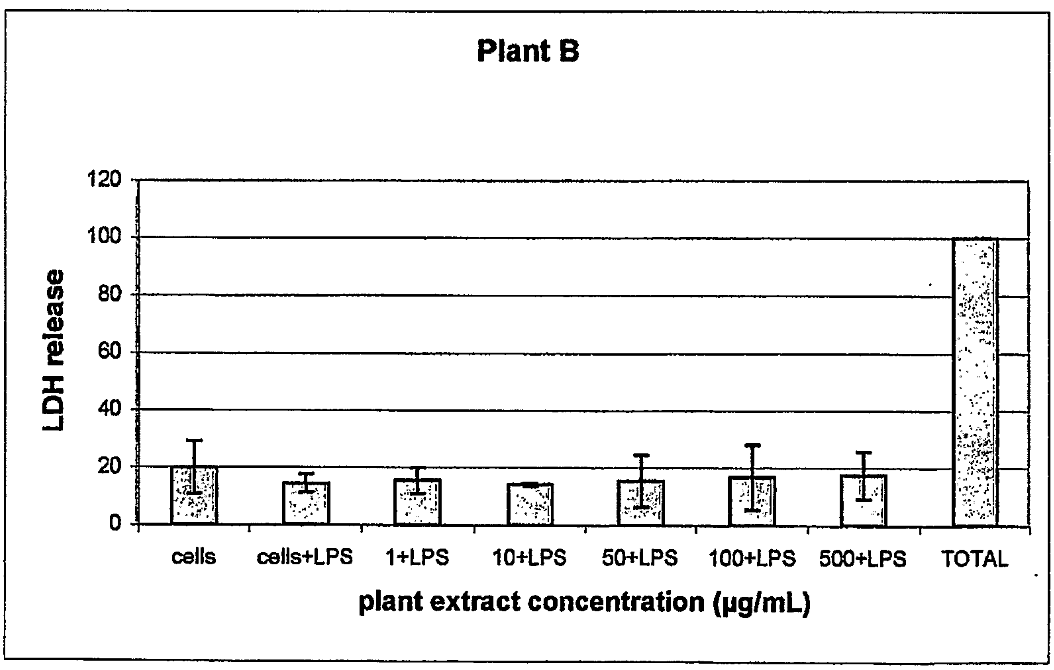
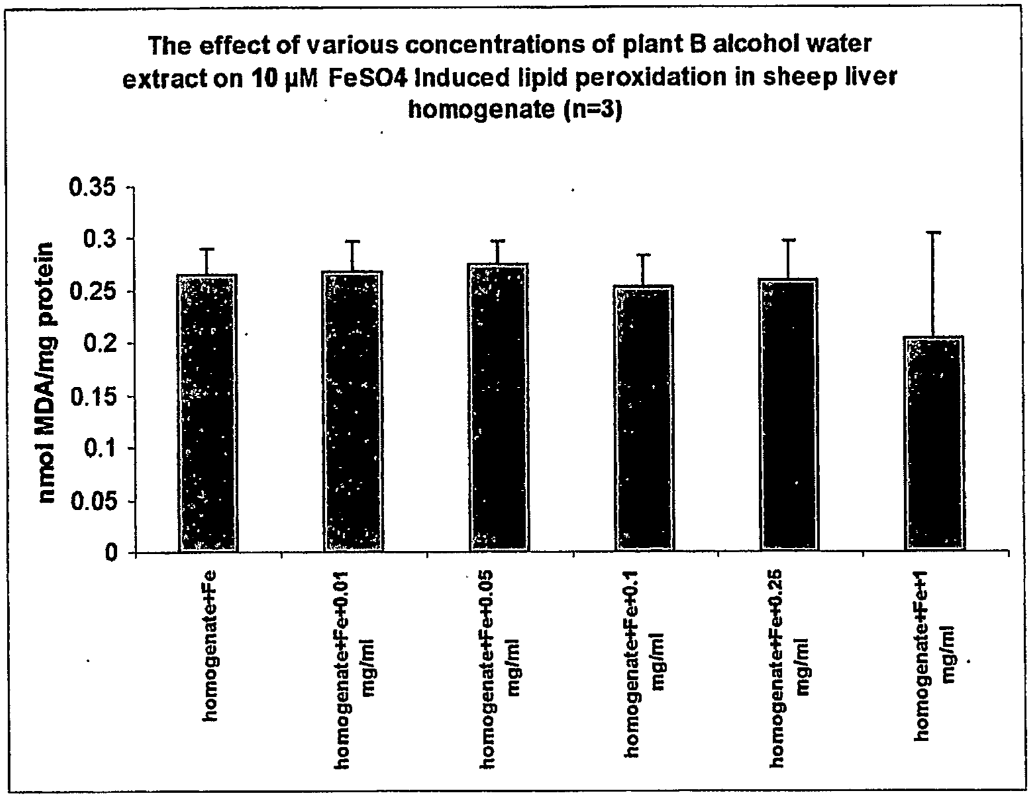


Figure 10: Amount of MDA versus plant extract concentration



iii. In Vitro Efficacy testing

The plant extract reduces the level of glucose in the bloodstream of diabetic patients as has been shown in the preliminary experiments discussed above. The complexity of diabetes type 2 as a disease makes it impossible to develop a single assay which would show in vitro, the efficacy that is found in humans (where the reduction in glucose levels is an overall effect without indication how this reduction is achieved.)

Several standard essays are available, which are based on measurement of the transport of glucose from the medium (blood) through the cell membrane into the cell. In addition, in vitro measurements have been performed on the adsorption of glucose through the walls of the small intestine into the bloodstream, giving an indication of glucose concentrations from the receiving end.

We have measured the effect of our plant extract on both these processes.

ix. Glucose adsorption through the intestine

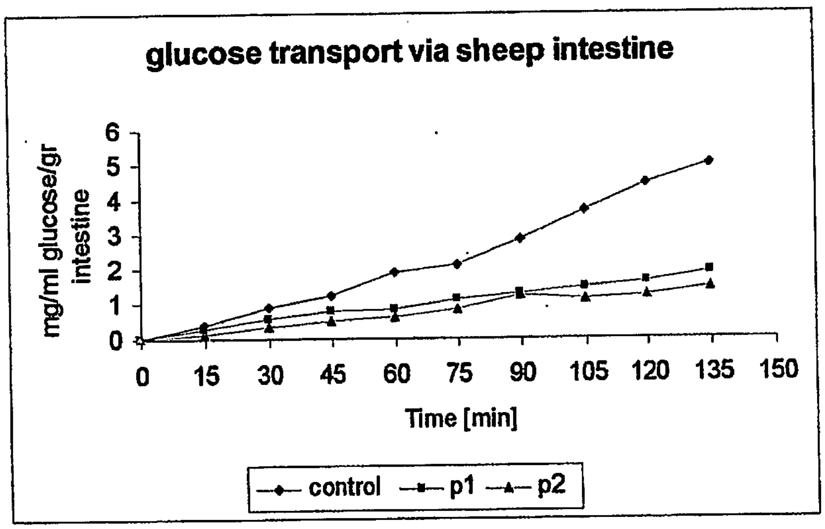
A 10 cm length of intestine was connected to a container which contain a Krebs solution with 100 mg/ml of D-glucos on one side and to collecting container from the other side, in such a way that the Krebs solution flows through the intestine. The flow rate of the solution into the

system was 50-55 ml/h. Th intestine was incubated in a thermostrated bath containing double distilled water at 37 °C.

Sugar is adsorbed through the intestine and transferred through the walls to the water bath. Samples from the bath were taken every 15 min. and the glucose concentration of the samples was tested according to the DNS method.

The graph below shows the effect of the plant extract on the adsorption of glucose. The measurements are normalized for the weight of the intestine sample, which is an indication of its protein content.

Figure 11: The effect of Plant B extract, on glucose adsorption through the intestine



The graph clearly shows, that the plant B extract partially inhibits the transfer of glucose through the intestine and this indicates a that a reduction of glucose levels in the bloodstream will occur.

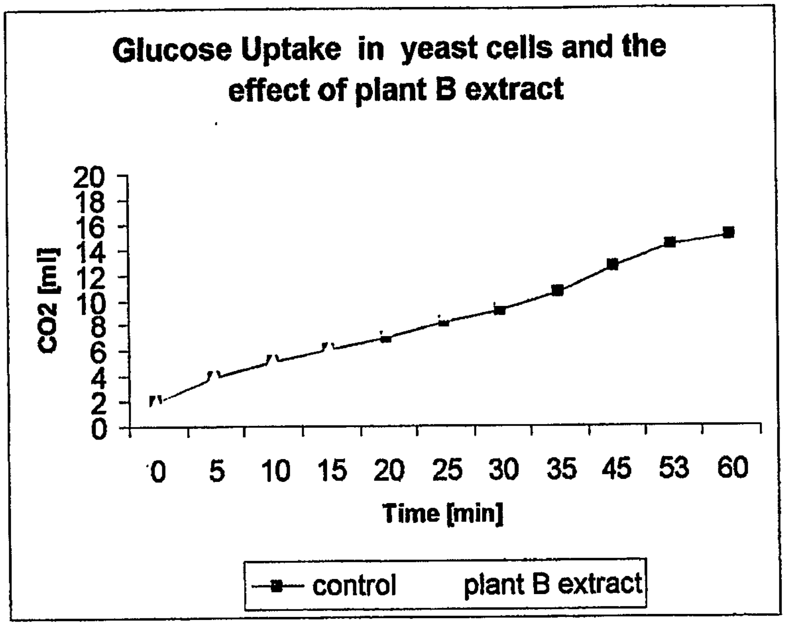
Glucose transport measurement into yeast cells.

This method, which has been in use for many years, exploits the evolution of CO2 by yeast cells as a measurement of activity, while the activity is a direct response to glucose concentration.

A measured amount of yeast c lls is kept under controll d circumstanc s and fed specific amounts of glucose, and the CO2 evolution is measured. The experiment is run in duplicate, whereby in the second experiment, in addition to glucose, also the plant B extract is added. The change in amount of CO2 evolved is a measure of the effect of plant B extract on the glucos transport into the cell.

See Figure 12 for an illustrative example.

Figure 12: Effect of Plant B extract on glucose transport into yeast cells



The graph shows that the plant extract increases the CO2 output of the yest cells, indicating and increased activity as a result of increased amounts of glucose being transported into the cell.

For our purpose, at this point, it is of importance that we succeed in mimicking as closely as possible, the effects of the plant extract on the glucose levels, both from a transport point of view as well as through effects on adsorption.

d. Standardization of Extract production

A standard procedure for the production of the extract has been developed. Such a standard procedure must guarantee a product of constant high quality.

5. Fractionation of Extract and Mechanistic analysis

In an effort to purify the extract and identify bioactive materials, a program for fractionation was initiated.

The activities may be divided in two main operations:

a. Purification of the ethanol/water extract through column chromatography followed by HPLC/MS and attempts to identify the products obtained. The chromatography is conducted with continuous monitoring of the fractions.

b. Repeated extractions with solvents of increasing polarity.

The range of solvents employed generally starts from hexane, through ethyl acetate, DCM, methanol and finally water. After each extraction, the plant material is filtered, dried and subjected to the ext extraction step.

The extracted materials were collected, dried and analyzed using both bioassays described above, as well as tlc and if justified HPLC.

Initial results are given in the table below.

Fraction	Intestine Assay	Yeast Assay
Ethanol/water	Impedes glucose transfer	Enhances glucose transport
Hexane	Impedes glucose transfer	No effect
Ethyl Acetate	Impedes glucose transfer	No effect
DCM	No effect	No effect
Methanol	No effect	Enhances Glucose Transport
Water	No Effect	Enhances Glucose Transport

The extraction with increasingly polar solvents resulted in a split of the biological effects of the original extract, with the non-polar extracts exhibiting an effect on the glucose transfer from the intestine to the bloodstream, while the polar extracts have an effect on the transport of glucose to the cells.

These results strongly indicate that at least two bioactive components are present in the extract, with effects on different stages of the metabolic path of glucose

6. Discussion

The plant extract from plant B has been shown to be biosafe and effective in the reduction of glucose levels in the bloodstream, both through in vitro experiments as well as through preliminary trials on human subjects, suffering from diabetes.

Purification of the extract and isolation of the active ingredients, allows a more in-depth study of the mechanism of the extract

In our in vitro experiments we have seen that the extract is active in the mechanism of transport of glucose into the cell and also has an influence of the adsorption of glucose

through the small intestine. This may indicate that there are at least two active ingredients present.

The repeated extraction procedure, using increasingly polar solvents, has results in a separation between fractions which are active in different stages of the metabolic pathway of glucose. With this we have shown that at least two bioactive components are present in the extracts and the ethanol/water extract which has been studied in depth, as discussed above, exhibits a combined biological effect of two bioactive components each of which functions distinctly different and separate.

This observation is of utmost importance in further work for the elucidation of the mechanistic aspects of the bioactivity of the plant, but at this point it suffices to state that the plant and the developed ethanol/water extract influence the metabolism of glucose in more than one way, which may allow options for a more regulated approach to glucose levels in the blood of diabetic patients.

6. Literature

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WHAT IS CLAIMED IS:

1. A composition for modulating glucose levels in an individual in need thereof, the composition comprising an alcohol and/or water extract of Portulaca Oleracea L., the composition being biosafe and effective in modulating said glucose levels, essentially as described herein.

2. A method of modulating glucose blood levels in an individual in need thereof comprising administering to the individual a therapeutic affective amount of an alcohol and/or water extract of Portulaca Oleracea L.

3. A method of identifying agents for modulating glucose levels, the method comprising:

(a) fractionating a Portulaca Oleracea L. to obtain multiple fractions; and

(b) identifying from said multiple fractions at least one fraction capable of modulating glucose levels, thereby identifying the agents for modulating glucose levels.

4. The method of claim 3, wherein said fractionating is effected by repeated extraction with solvent of increasing polarity.

5. A composition for modulating glucose levels in an individual in need thereof, the composition comprising an extract of Portulaca Oleracea L., said extract being capable of inhibiting glucose transfer to the circulation and/or increasing glucose transfer to cells.

6. The composition of claim 5, wherein said extract is a polar extract

7. The composition of claim 5, wherein said extract is a non-polar extract.